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Subunit Specificity of Mutations That Confer Resistance to Nonnucleoside Inhibitors in Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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We constructed plasmid vectors that simultaneously express both the p66 and p51 subunits of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) in *Escherichia coli*. These vectors allow us to generate HIV-1 RT heterodimers in which either the p66 or the p51 subunit has the wild-type sequence and the other subunit has a specific amino acid substitution. We used these vectors to express HIV-1 RT heterodimers containing several different amino acid substitutions reported to confer resistance to nonnucleoside inhibitors. Most of the amino acid substitutions conferred resistance to nonnucleoside inhibitors R86183 (TIBO) and TSAO-m^{3}T only when present in the p66 subunit of the p66-p51 heterodimer; heterodimers that contained a wild-type p66 subunit and a mutant p51 subunit remained sensitive to the inhibitors. However, there was one mutation, E138K, that conferred drug resistance when the mutation was present in the p51 subunit. The corresponding heterodimer with the E138K mutation in the p66 subunit and a wild-type p51 subunit remained sensitive to the inhibitors. Analysis of the three-dimensional structure of HIV-1 RT indicated that residue 138 of the p51 subunit is in the nonnucleoside inhibitor-binding pocket while residue 138 of the p66 subunit is not. The mutagenesis results, combined with structural data, support the idea that the nonnucleoside inhibitors exert their effects by binding to a hydrophobic pocket in the RT heterodimer and that mutations which give rise to drug resistance directly interfere with the interactions between the nonnucleoside inhibitors and HIV-1 RT.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) converts the single-stranded RNA that makes up the viral genome into double-stranded DNA by using a DNA polymerase activity which can copy either RNA or DNA templates, and an RNase H activity which degrades RNA only when it is part of a DNA-RNA heteroduplex (43, 45). In the virion HIV-1 RT is a heterodimer consisting of a 66-kDa subunit (designated p66) and a 51-kDa subunit (designated p51). The p66 subunit contains both the DNA polymerase domain and the RNase H domain. The smaller p51 subunit is a proteolytic cleavage product of the p66 protein or of a larger precursor and contains the DNA polymerase domain but lacks the RNase H domain (18, 23, 24, 29, 37). Since the two subunits are derived from the same coding region in the viral genome, a single mutation in the RT-coding region yields a heterodimer containing two amino acid substitutions, one in each subunit. However, since the structures of the p66 and p51 subunits within the RT heterodimer are different, these two amino acid substitutions within the heterodimer are nonequivalent (1, 26, 27, 33, 41). The crucial role of RT in the life cycle of HIV-1 has made it the target for various anti-AIDS drugs, including nucleoside analogs and the structurally diverse nonnucleoside inhibitors. However, HIV-1 appears to be genetically flexible and HIV-1 strains resistant to each of these inhibitors have been obtained. The basis for drug resistance appears to be amino acid substitutions within RT (for reviews, see references 16, 17, and 38).

We constructed plasmids that express a p51 subunit and a p66 subunit from two separate coding regions. These plasmids allow us to generate heterodimers containing one mutant subunit and one wild-type subunit. These vectors were used to express heterodimers containing amino acid substitutions reported to confer resistance to nonnucleoside inhibitors in either the p66 or the p51 subunit. These heterodimers were used to test which subunit confers resistance to TIBO class nonnucleoside drugs R86183 and R82913 and nonnucleoside drug TSAO-m^{3}T. The heterodimers were also used to look for differences in the interactions of these various inhibitors with the different resistance mutations.

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MATERIALS AND METHODS

Construction of pUC12N/p66(His) and pUC12N/p51(His). Vectors pUC12N/p66(His) and pUC12N/p51(His) are derived from the expression vector pUC12N (34, 44). pUC12N (*Not*I-*Pme*I) is a modification of pUC12N and contains unique *Not*I and *Pme*I sites between the plasmid origin of replication and the *lacZ* coding region.

Clone RT(66) has been previously described (24, 25). Clone RT(His) is a modification of RT(66) that contains, in addition to the normal RT-coding region, an additional six histidine codons at the 3′ end of the coding region. Clone p51(AH) contains a termination codon 3′ of the codon for amino acid 440, which is the site where HIV-1 protease cleaves to produce p51 in virions. p51(His) is similar but contains an additional six histidine codons before the termination codon. The recognition sequence for the *Nco*I restriction endonuclease at the initiator ATG of RT(His) and p51(His) was removed by altering the sequence from CC ATG GTT to CC ATG CTT. After the *Nco*I site was removed, the *Hin*dIII site was removed by digestion with *Hin*dIII and filling in of the 5′ overhang with the Klenow fragment of DNA polymerase I. The blunt-ended DNA was ligated to *Not*I linkers, digested with restriction

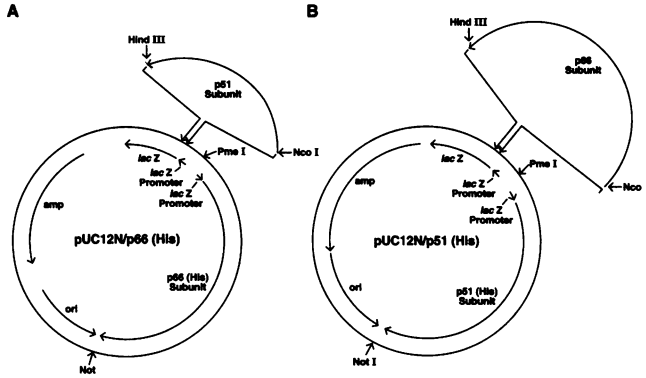

FIG. 1. Maps of subunit coexpression vectors pUC12N/p66(His) (A) and pUC12N/p51(His) (B). These vectors are derivatives of protein expression vector pUC12N (34, 44). pUC12N/p66(His) expresses the wild-type p66 subunit with a six-histidine extension on the carboxyl terminus of the protein. pUC12N/p51(His) expresses the wild-type p51 subunit, also with a six-histidine extension on the carboxyl terminus of the protein.

endonuclease *Not*I, and recircularized. These RT(His) and p51(His) clones, which lacked both the *Nco*I and *Hin*dIII recognition sites and contained the *Not*I recognition sequence 3′ of the coding region, were digested with restriction endonucleases *Not*I and *Bst*UI. The 1.9-kb fragment from the RT(His) clone and the 1.5-kb fragment from the p51(His) clone were isolated and ligated to *Not*I-*Pme*I-digested pUC12N (*Not*I-*Pme*I) to give clones pUC12N/p66(His) and pUC12N/p51(His) (Fig. 1). All clones were transformed into *Escherichia coli* DH5α (GIBCO BRL), which is a *recA* mutant.

Construction of clones expressing heterodimers. Individual drug resistance-encoding mutations were prepared in RT(66) clones by the method of *Bsp*MI cassette mutagenesis as previously described (10, 11). These clones express only the p66 subunit of HIV-1 RT. To construct a clone expressing a mutant p66 subunit and a wild-type p51 subunit, the plasmid expressing the appropriate mutant p66 subunit was digested with restriction endonucleases *Nco*I and *Hin*dIII, sites for which flank the RT-coding region. The 1.7-kb fragment was gel purified and ligated to *Nco*I-*Hin*dIII-digested pUC12N/p51(His).

p51(AH) contains an *Nco*I site at the initiator ATG and a *Hin*dIII site 3′ of the termination codon. p51(AH) also contains a unique *Asp*718 site within the p51-coding region, approximately 40 bp 5′ of the termination codon. To obtain a clone expressing a mutant p51 subunit, p51(AH) was digested with restriction endonucleases *Nco*I and *Asp*718 and the 2.7-kb fragment was gel purified. This fragment contains the pUC12N plasmid sequences, the last 40 bp of the coding region for the p51 subunit, and the termination codon. The mutant RT(66) clones were also digested with restriction endonucleases *Nco*I and *Asp*718, and the 1.2-kb fragment was gel purified. This fragment contains the 5′ end of the RT-coding region, and when it is ligated to the fragment from p51(AH), a clone expressing a mutant p51 subunit is generated. To construct a clone expressing a mutant p51 subunit and a wild-type p66 subunit, a clone which expressed a mutant p51(AH) subunit was digested with restriction endonucleases *Nco*I and *Hin*dIII. The 1.3-kb fragment was gel purified and ligated to *Nco*I-*Hin*dIII-linearized pUC12N/p66(His).

RT assays. RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H activities were assayed in *E. coli* extracts as previously described (9–11). TIBO class drugs R86183 and R82913 were gifts from the Janssen Research Foundation. TSAO-m^{3}T was obtained from Jan Balzarini.

RESULTS

Plasmids pUC12N/p66(His) and pUC12N/p51(His) each contain two *lacZ* promoters oriented in opposite directions (Fig. 1) and are similar in concept to the plasmid constructed by Müller et al. (32). However, unlike the plasmids developed by Müller et al., the plasmids we developed make it simple to produce heterodimers containing specific mutations in either the p51 or p66 subunit. As described in Materials and Methods, the DNA fragments encoding mutant p66 subunits were inserted into the polylinker region of plasmid pUC12N/p51(His). These plasmids, e.g., Y181I/p51(His), expressed a mutant p66 subunit and a wild-type p51 subunit (Fig. 1B). In parallel, regions encoding mutant p51 subunits were inserted into the polylinker region of plasmid pUC12N/p66(His). These plasmids, e.g., p66(His)/Y181I(p51), expressed a wild-type p66 subunit and a mutant p51 subunit (Fig. 1A). As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, the resulting *E. coli* strains expressed high levels of the two subunits and the expression levels of the two subunits appeared to be equivalent (data not shown).

In the initial experiments, five mutants were analyzed with TIBO derivative R86183 (36) as the inhibitor (Fig. 2; Table 1). Amino acid substitutions Y181I, K103N, Y188L, V106A, and E138K were present either in the p66 subunit [e.g., Y181I/p51(His)] or in the p51 subunit [e.g., p66(His)/Y181I(p51)]. In all cases, the other subunit had a wild-type sequence. The wild-type heterodimer was expressed by cloning a wild-type p51-coding region into vector pUC12N/p66(His) to generate clone p66(His)/p51. This wild-type heterodimer was used as a control in these experiments. Four of the amino acid substitutions (Y181I, K103N, Y188L, and V106A) showed resistance to R86183 when the RT heterodimer contained the amino acid substitution in the p66 subunit (Fig. 2; Table 1). The corresponding RT heterodimers that contained a wild-type p66 subunit and a mutant p51 subunit were as sensitive to the inhibitor as wild-type HIV-1 RT. We also tested amino acid substitutions Y181C, Y188C, and Y188H in this system, and the results were similar to the results obtained with the Y181I and Y188L mutations (data not shown).

Amino acid substitution E138K has the opposite subunit specificity. Heterodimers that contained a mutant p51 subunit and a wild-type p66 subunit were resistant to R86183 (Fig. 2E; Table 1). However, RT heterodimers that contained a mutant p66 subunit and a wild-type p51 subunit remained completely sensitive to the drug. Interestingly, it has been reported that the presence of the E138K mutation was specific for the TSAO family of inhibitors and, in vivo, this mutation did not show cross-resistance to other nonnucleoside inhibitors, including TIBO compound R82913 (3–8). Our data show that the E138K mutation is able to confer partial resistance to another nonnucleoside drug, R86183. Moreover, a recent HIV-1 clinical isolate from a patient being treated with R82913 contained an RT with amino acid substitutions E138A and E169D, which showed an 11-fold reduction in sensitivity to the compound (42). We tested the p66(His)/p51, E138K/p51(His), and p66(His)/E138K(p51) heterodimers against R82913 and found that while the p66(His)/E138K(p51) heterodimer has limited resistance to R82913, this effect is not as pronounced as that seen for R86183 (Table 1).

We also tested whether these mutant HIV-1 RTs would be

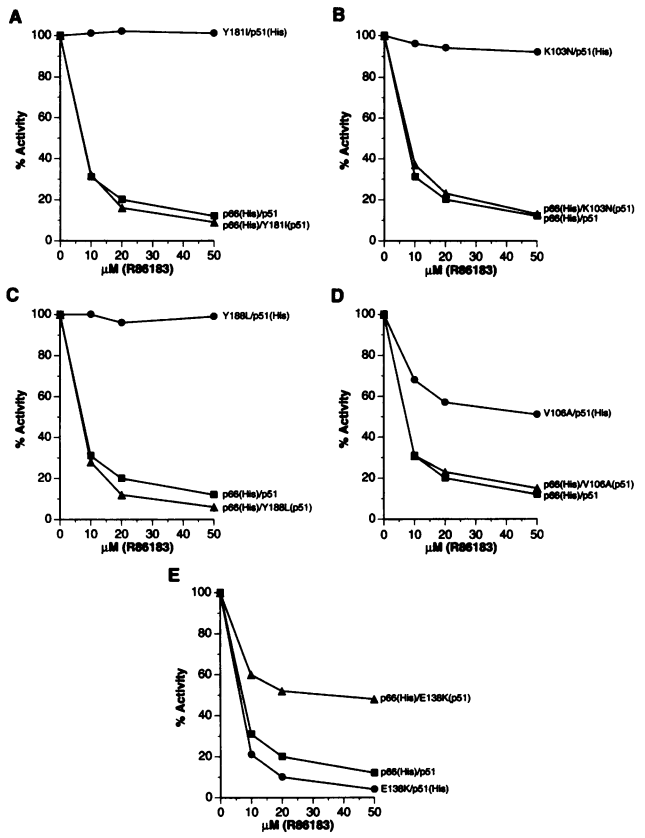

FIG. 2. Subunit specificity of resistance to nonnucleoside inhibitor R86183 by mutants Y181I (A), K103N (B), Y188L (C), V106A (D), and E138K (E). Each panel shows results obtained with the wild-type heterodimer [p66(His)/p51] as a control, a heterodimer with the mutation in the p66 subunit and a wild-type p51 subunit [e.g., Y181I/p51(His)], and a heterodimer with the amino acid substitution in the p51 subunit and a wild-type p66 subunit [e.g., p66(His)/Y181I(p51)]. The assays were all repeated at least twice with extracts derived from separate bacterial transformations. The separate assays gave a less than 5% variation in the experimental data.

resistant to the N^3-methylthymine derivative of TSAO (TSAO-m^3T). Again, amino acid substitutions Y181I, K103N, Y188L, and V106A conferred resistance to TSAO-m^3T when they were present in the p66 subunit of the heterodimer. Heterodimers containing these mutations in the p51 subunit and containing a wild-type p66 subunit remained sensitive (Table 1). However, mutation K103N was more sensitive to TSAO-m^3T than to R86183 (Table 1). The roles of the subunits are reversed for amino acid substitution E138K. This mutation conferred resistance to TSAO-m^3T only when it was present in the p51 subunit of the heterodimer. When the mutation was present only in the p66 subunit, the heterodimer was as sensitive to the inhibitor as was the wild-type enzyme (Table 1).

Other amino acid substitutions have been reported to be involved in resistance to nonnucleoside inhibitors. Two of these mutations, L100I and V179D, were tested in the coexpression system against TSAO-m^3T and R86183. Mutation L100I was similar to most of the other amino acid substitutions analyzed in that it conferred resistance to R86183 only when present in the p66 subunit (Table 1). Neither the enzyme containing an L100I mutation in the p66 subunit nor the enzyme with the mutation in the p51 subunit was resistant to TSAO-m^3T (Table 1), in agreement with results obtained by

TABLE 1. Summary of degrees of resistance conferred by various amino acid substitutions to different nonnucleoside inhibitors[a]

Mutation (subunit)	Degree of resistance (%) to:		
	R86183	R82913	TSAO-m^3T
None (wild type)	0	0	0
L100I (p66)	100	ND	0
L100I (p51)	0	ND	0
K103N (p66)	85	ND	25
K103N (p51)	0	ND	0
V106A (p66)	40	ND	40
V106A (p51)	2	ND	3
E138K (p66)	0	0	0
E138K (p51)	35	10	35
V179D (p66)	2	25	ND
V179D (p51)	0	0	ND
Y181I (p66)	100	ND	100
Y181I (p51)	0	ND	0
Y188L (p66)	100	ND	50
Y188L (p51)	0	ND	0

[a] The heterodimers contain the amino acid substitution in the indicated subunit, while the other subunit had a wild-type sequence. The numbers indicate the residual activity relative to the level of activity in the absence of the inhibitor. At the highest level of inhibitor used in the assays, the wild-type heterodimer retained approximately 10% of its RNA-dependent DNA polymerase activity (Fig. 2). Since the wild-type heterodimer is not resistant to the inhibitors, this level was considered 0% resistance and the data from the mutant enzymes were normalized by using this value. The assays were repeated at least twice with extracts derived from separate bacterial transformations.

[b] ND, not done.

Balzarini et al. (5, 6). When V179D mutants were analyzed with inhibitor R86183, neither of the V179D enzymes showed significant resistance (Table 1). The V179D mutation was originally selected in vivo with TIBO derivative R82913 (4, 13, 42). When the heterodimers were challenged with this second TIBO derivative, resistance could be detected when the mutation was present in the p66 subunit; no effect was seen when the mutation was present only in the p51 subunit (Table 1).

DISCUSSION

The structure of the HIV-1 RT heterodimer in complex with the nonnucleoside inhibitor nevirapine (27, 41) or a derivative of nonnucleoside inhibitor α-anilinophenylacetamide (18a) shows that the drugs are bound in a hydrophobic pocket near the polymerase active site (Fig. 3). Other studies have suggested that all of the nonnucleoside inhibitors bind within this hydrophobic pocket (14, 19, 21, 22, 40). The pocket is bounded by two β sheets and the β5a-β6 connecting loop of the p66 palm subdomain (Fig. 4; 33, 41), and most of the amino acid substitutions reported to confer resistance to nonnucleoside inhibitors (for reviews, see references 16, 17, and 38) are located on these β sheets (Fig. 4). The corresponding amino acid substitutions in the p51 subunit are not close to the hydrophobic pocket (Fig. 3; 1, 18a, 26, 27, 33, 41). One of the β sheets, containing β9, β10, and β6, forms the floor of the binding pocket and contains amino acid residues V-106, V-108, V-179, Y-181, Y-188, and G-190, as well as the catalytic triad of aspartic acids (D-110, D-185, and D-186) which are essential for polymerization (Fig. 4; 33, 41). The other β sheet, contain-

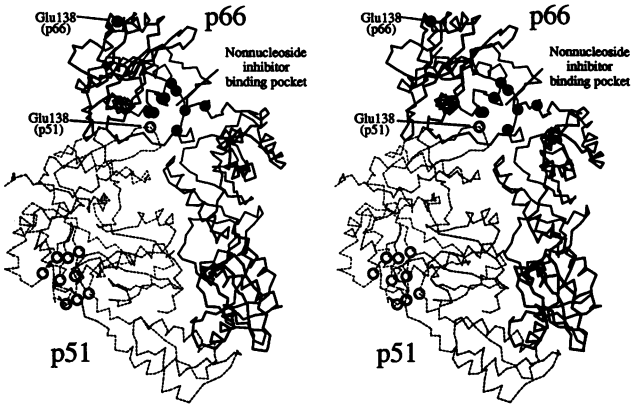

FIG. 3. Stereo view of the Cα backbone trace of the HIV-1 RT p66-p51 heterodimer taken from the ternary complex of HIV-1 RT–double-stranded DNA–Fab28 (26) showing the location of the nonnucleoside inhibitor-binding pocket and the locations of the nonnucleoside drug resistance-encoding mutations in both the p66 and p51 subunits. The figure was generated with MOLSCRIPT (28). The Cα backbone of the p66 subunit is shown as a solid black line, while the Cα backbone of the p51 subunit is shown as a dashed line. The locations of the nonnucleoside drug resistance-encoding mutations in the p66 subunit are shown as filled circles; the locations of these mutations in the p51 subunit are shown as open circles. Most of the mutations confer drug resistance only when they are present in the p66 subunit and consequently lie near the nonnucleoside drug-binding pocket. Mutation E138K is an exception. This mutation in the p66 subunit is located at the tip of the fingers subdomain and is not located near the drug-binding pocket; the corresponding mutation in the p51 subunit is near and forms part of the drug-binding pocket. The drug resistance of mutation E138K is manifested through the change in the p51 subunit.

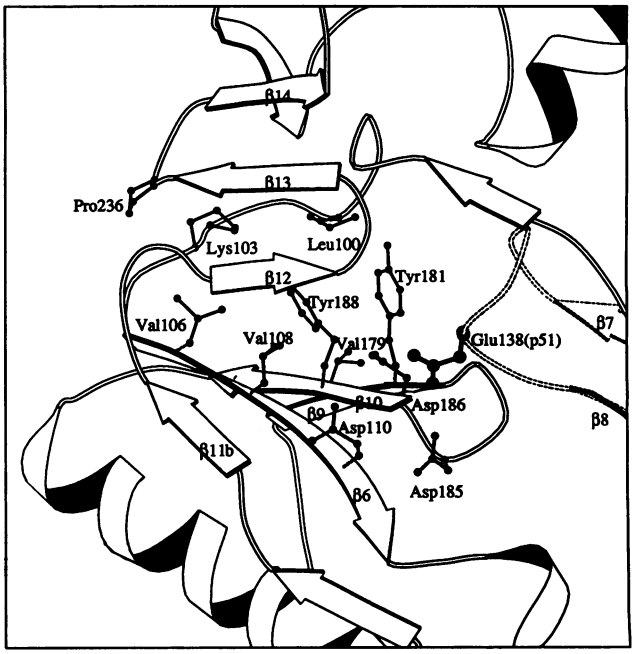

FIG. 4. Close-up of the nonnucleoside inhibitor-binding pocket taken from the structure of the HIV-1 RT–double-stranded DNA–Fab28 complex. Shown are the secondary structural elements forming the drug-binding pocket, the side chains of some amino acids where nonnucleoside drug-resistant mutations occur, and the side chains of the three essential aspartic acid residues at positions 110, 185, and 186 at the polymerase active site. As shown in Fig. 3 and discussed in the text, the β7-β8 loop of p51 (dashed lines) is adjacent to the drug-binding pocket and residue E-138 from p51 (in the thicker ball-and-stick model) forms part of the drug-binding pocket. Dashed lines in the amino acid side chains represent imaginary connections to the β strands.

ing the β12-β13 hairpin (also known as the primer-grip) and β14, forms the roof of the binding pocket and contains residue P-236. The β5b-β6 connecting loop contains amino acid residues L-100 and K-103. Therefore, from the structural analysis it appears that most of the mutations conferring resistance to the nonnucleoside inhibitors should exert their effects only when they are present in the p66 subunit and should exert no effect when present in the p51 subunit. Structural analysis indicated that amino acid substitution E138K may be an exception (Fig. 4; 33). A segment of the p51 subunit, the connecting loop between β7 and β8, also contributes to the pocket and includes residue E-138 (Fig. 4; 33, 41). In the p66 subunit, the β7-β8 loop lies near the tip of the fingers subdomain and is not close to the hydrophobic pocket (Fig. 3), and it has been suggested that the E138K mutation exerts its effects through the change in the p51 subunit rather than through the change in the p66 subunit (33). Our biochemical results confirm the predictions made on the basis of structural data. We show that most of the amino acid substitutions involved in nonnucleoside drug resistance confer resistance if, and only if, they are present in the p66 subunit of the HIV-1 RT heterodimer. A heterodimer containing a mutant p51 subunit and a wild-type p66 subunit remained sensitive to the inhibitors. As predicted, the exception was mutant E138K, which conferred resistance to the nonnucleoside drugs only when it was present in the p51 subunit. These results indicate that the nonnucleoside resistance mutations directly alter the hydrophobic pocket. These alterations could affect residues directly involved in binding interactions, distort the pocket so that the inhibitor can no longer bind effectively, or cause stearic hindrance which could prevent the inhibitor from entering the pocket.

The mutant heterodimers produced by the coexpression plasmids also allow us to study differences in the interactions of the various inhibitors with the various mutations. Mutations conferring resistance to one type of nonnucleoside inhibitor do not necessarily confer complete resistance to other types of nonnucleoside inhibitors, indicating that the compounds may bind differently in the pocket and may interact with different amino acids within the pocket (2–9, 13, 20, 21, 31). The TIBO class of compounds and the TSAO class of compounds are structurally different from each other and from the other nonnucleoside inhibitors (for reviews, see references 16, 17, and 38). Although the genetic and biochemical data show that TIBO and TSAO compounds bind to the same hydrophobic pocket as nevirapine and α-anilinophenylacetamide, the effects of the TIBO and TSAO compounds on HIV-1 RTs with various amino acid substitutions are distinct (Table 1). Both inhibitors appear to interact with the tyrosine residues at positions 181 and 188, in agreement with data obtained by other groups (3–6, 13, 15, 30, 35, 39, 40, 42). However, there is a striking difference between the ways these inhibitors respond to amino acid substitutions at positions 100 and 103 (Table 1). RTs containing amino acid substitutions L100I and K103N show greatly increased resistance to TIBO compounds but only partial or no resistance to the TSAO derivative. Residues 100 and 103 lie near each other on the connecting loop between β5b and β6 (Fig. 4; 33, 41) and appear to interact with TIBO compounds but only partially or not at all with TSAO compounds.

We also detected differences in inhibitor responses to mutations V179D and E138K (Table 1). TIBO compounds R82913 and R86183 interacted differently with the V179D and E138K mutations. Structurally, the two compounds are almost identical; R82913 is a 9-chloro derivative, while R86183 is an 8-chloro derivative (36). However, these slight differences have a large effect on the abilities of these two compounds to inhibit the replication of wild-type HIV-1 (36) and the Rauscher murine leukemia virus (12) in vivo. R86183 was 10-fold more effective in inhibiting HIV-1 replication than was R82913, but R82913 could inhibit the replication of the murine retrovirus while R86183 could not. Heterodimers containing the V179D mutation in the p66 subunit did not confer resistance to R86183 but were able to confer resistance to R82913. The results were reversed for heterodimers containing the E138K mutation in the p51 subunit. These heterodimers did not show resistance to R82913 but showed resistance to R86183. Since the relative levels of resistance to the two inhibitors are reversed for the two mutants, it is not simply a matter of one compound being a better inhibitor. These data suggest that the change in the position of the chloro group may alter how the compounds bind within the pocket and that R86183 interacts with the amino acids in the pocket somewhat differently from R82913. Residue V-179 of the p66 subunit lies close to residue E-138 of the p51 subunit in the three-dimensional structure of the HIV-1 RT heterodimer (Fig. 4; 33, 41). Because the only difference between the structures of these TIBO compounds is in the position of the chloro moiety, it is possible that when these compounds are in the hydrophobic pocket, the portion of the inhibitors which carries the chloro groups lies near residues E-138 and V-179. While this is a plausible model, other interpretations of the data are possible and resolution of the issue requires structural analysis.

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